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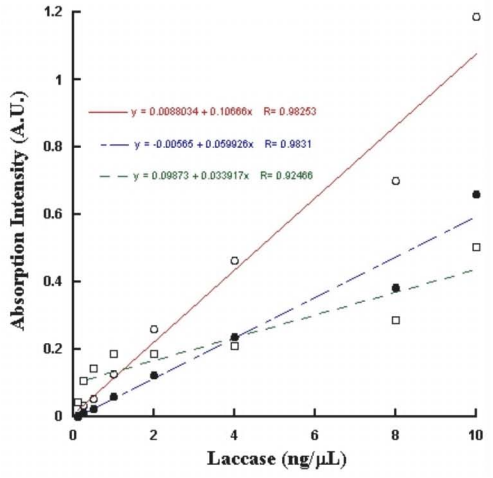
Multi-mode Spectroscopic High Throughput Screening (HTS) of Phenols and Monolignols

Komandoor Achyuthan*, Blake Simmons, Paul Adams, and Anup
Singh Joint BioEnergy Institute (JBEI), Emeryville, CA 94608 *
Presenter (Email: kachyut@sandia.gov)

Phenolic structure is a common motif among the monolignols, coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol. We are developing sensitive, rapid, multiplexed and high throughput screening (HTS)-compatible UV-Vis and fluorescence spectroscopic assays for phenols and monolignols within 96-and 384-well microplates. We used p-cresol as a model for the phenols and coniferyl alcohol as a prototype monolignol. We employed the fungal (Trametes versicolor) laccase enzyme to oxidize p-cresol and coniferyl alcohol, and thereby expanded the spectroscopic properties of these molecules. Laccases are involved in lignin degradation. Our choice of laccase is especially relevant, since the enzyme was purified from white rot basidomycetes that are efficient degraders of lignins and widely studied for biofuels.

We supply a menu of spectroscopic options for the HTS of laccase oxidation of p-cresol through multiple modes of detection. Laccase activity was monitored kinetically at pH 4.5 by absorption changes at 250nm, 274nm or 297 nm, and in endpoint mode by the bathochromic shift in absorption to 326nm. Laccase oxidation of p-cresol was also detected by product fluorescence at 425nm after excitation at 262nm or 322nm. We optimized the kinetic parameters for p-cresol oxidation (pH optimum 4.5-5.1; 37oC; Km = 2.2mM) resulting in laccase limits of detection and quantization (LOD, LOQ) of 25pg/µL and 75pg/µL, respectively (~360pM; 25ppb). The p-cresol LOD was 8µM with a potential for further improvements in sensitivity. A key advantage of our assay is that laccase catalysis could be interrogated using multi-mode spectroscopy under acidic or basic conditions, in real time or endpoint modes.



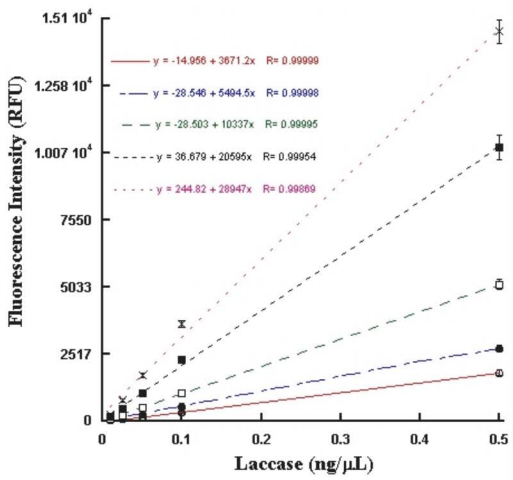


Figure-1 (left panel). Absorption changes of laccase-catalyzed p-cresol oxidation. Laccase oxidation of 1mM p-cresol was in pH 4.5 buffer for 10 min. Absorption changes at 250nm (open circles), 297nm (closed circles) and 326nm (open squares) were measured as described above. *Figure-2 (right panel).* Laccase dose-response curves from 425nm fluorescence emission (excitation = 322nm). Reaction conditions were as described for Figure-1. Fluorescence was monitored for 2.5 min. (open circles), 5 min. (closed circles), 10 min. (open squares), 20 min. (closed squares) and 30 min. (crosses). All reactions were linear (r2 > 0.99). We similarly characterized the spectroscopic properties of coniferyl alcohol in seven different solvents. Three isosbestic wavelengths were identified at 240nm, 242nm and 262nm

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between NaOH and the six solvents. A S/B of ~50 with 500µM coniferyl alcohol indicated assay sensitivity. The excitation spectrum was broad (270 – 335nm) and overlapped with absorption spectrum, as expected. Fluorescence emission was between 360 – 500nm with peak at 416 – 420nm. Fluorescence spectroscopy gave 1µM of coniferyl alcohol detection sensitivity. Unlike p-cresol, a fluorescence quench was observed following laccase oxidation of coniferyl alcohol.

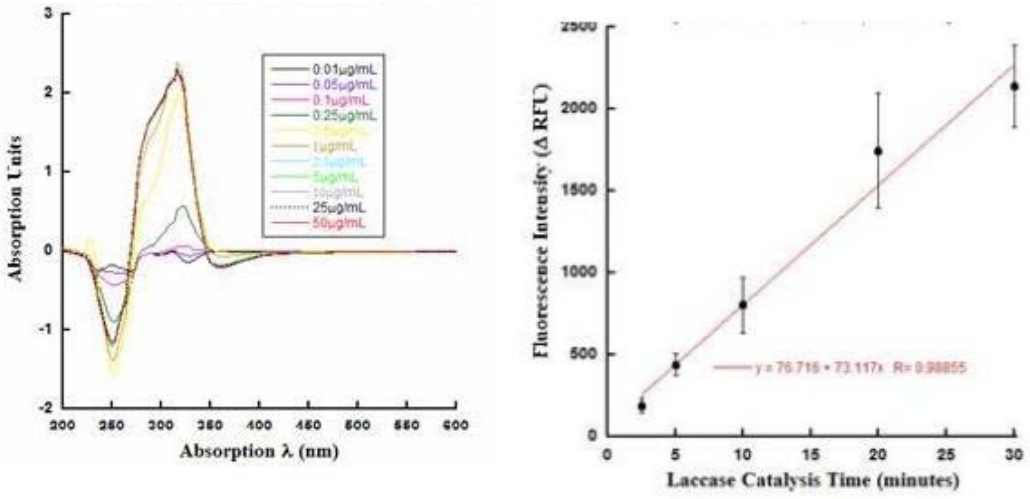

Figure-3 (left panel). UV-Vis Difference Spectra of laccase-catalyzed oxidation of Coniferyl alcohol. Increasing concentrations of laccase were reacted in pH 4.5 assay buffer for 60 minutes with 1mM Coniferyl alcohol. The absorption changes taking place over the wavelength of 200 – 600nm are shown along with the concentrations of laccase used as inset. *Figure-4 (right panel).* Laccase reaction kinetics from 416nm fluorescence emission (excitation = λ310 or 320nm). Reaction conditions were as described for Figure-1. Laccase activity was detected rapidly, in as little as 2.5 minutes towards the Coniferyl alcohol substrate.

In conclusion, we demonstrated sensitive, rapid, HTS-compatible fluorescence "turn on" and "turn off" spectroscopic assays for phenols and monolignols. Orthogonal interrogation and ratiometric analysis are key features of our assay, enabling high specificity and minimizing interferences during compound library screening. A portion of this work has been accepted for publication (below). We plan to expand our investigations to include the remaining two monoligonols: sinapyl and p-coumaryl alcohols.

Publication: K. E. Achyuthan, et al, Comb. Chem. HTS, 2009 (Accepted; In Press).

Acknowledgements:
This work was funded by the DOE Joint BioEnergy Institute (http://www.jbei.org) supported by the U. S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy. Sandia is a multi-program laboratory operated by Sandia Corporation, a Lockheed Martin Company for the United States Department of Energy's National Nuclear Security Administration under contract # DE-AC04-94AL85000.